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Role of the locus and of the resistance gene on gene amplification frequency in methotrexate resistant *Leishmania tarentolae*

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ABSTRACT

The protozoan parasite *Leishmania* resists the antifolate methotrexate (MTX) by amplifying the R locus dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene, the H locus *ptr1* pterin reductase gene, and finally by mutation in a common folate/MTX transporter. Amplification of *dhfr-ts* has never been observed in *Leishmania tarentolae* MTX resistant mutants while *ptr1* amplification is common. We have selected a *L.tarentolae ptr1* null mutant for MTX resistance and observed *dhfr-ts* amplification in this mutant demonstrating that once a preferred resistance mechanism is unavailable, a second one will take over. By introducing the *ptr1* gene at the R locus and the *dhfr-ts* gene at the H locus by gene targeting, we investigated the role of the resistance gene and the locus on the rate of gene amplification. Transfection studies indicated that *ptr1* gave higher levels of MTX resistance than *dhfr-ts*. Consistent with this, when *ptr1* was present as part of either the H locus or the R locus it was invariably amplified, while *dhfr-ts* was only amplified when *ptr1* was inactivated. When *dhfr-ts* was present in a *ptr1* null background on both the H locus and the R locus, amplification from the H locus was more frequent suggesting that both the gene and the locus are determining the frequency of gene amplification in *Leishmania*.

INTRODUCTION

Reduced folates serve as co-factors in a variety of one carbon transfer reactions such as in the formation of thymidylate. The enzyme dihydrofolate reductase (DHFR) is the key enzyme for providing reduced folates to the cell and is an important target of chemotherapeutic drugs known as antifolates. In protozoa and plants, DHFR is fused to thymidylate synthase (TS) resulting in a bifunctional DHFR-TS enzyme (1,2).

Chemotherapy is the only effective way to control the infection caused by the protozoan parasite *Leishmania* and the first line drug consists in pentavalent antimony. Although the antifolate methotrexate (MTX) is not useful for the treatment of *Leishmania*, extensive work has been carried out on the mechanism of MTX resistance in *Leishmania* (reviewed in 2,3). These studies have been useful to pinpoint novel putative intracellular targets suggesting that antifolates could be useful against *Leishmania* (1,2) and have led to several insights on the mechanisms of gene amplification and rearrangements in this parasite (4,5).

The first mutation characterized in *Leishmania* MTX resistant mutants was the amplification of the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene as part of the R locus (6). In addition to the *dhfr-ts* gene, another locus was found amplified in MTX resistant *Leishmania* and was named the H locus (7). The gene on the H locus responsible for MTX resistance was characterized and its product, PTR1, showed sequence similarities to the short chain dehydrogenase/reductase family (8,9). PTR1 can reduce pterins and folates and when overexpressed can reduce sufficient dihydrofolate into tetrahydrofolate hence rendering the cell resistant to DHFR inhibition (10–15). *Leishmania* have a common folate/MTX transporter and mutations in the gene for this transporter can also lead to MTX resistance (16–21). Amplification of *dhfr-ts* in MTX resistant *Leishmania* has only been observed in *Leishmania major* (4,17,22) but never in *Leishmania donovani* or *Leishmania tarentolae* or in other species selected for MTX resistance. In contrast, *ptr1* amplification and reduced uptake of the drug have been observed in every *Leishmania* species selected for MTX resistance. The reason for the lack of amplification of *dhfr-ts* in species other than *L.major* is unknown.

The availability of gene transfection technology in *Leishmania* has facilitated the study of gene amplification mechanisms. It made possible the testing of the hypothesis (4,23,24) that extrachromosomal amplicons are formed by recombination through homologous direct or inverted repeats (25) and that linear amplicons can be the precursor of circular amplicons (26). In this study we have addressed the effect of the locus and of the gene on amplification frequency following MTX selection in *Leishmania*.

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MATERIALS AND METHODS

Cell lines and cultures

The *L.tarentolae* wild-type cell line TarII and its *ptr1* null mutant were described previously (23,27). Cell lines were grown in SDM-79 or in M199 media. Mutants derived from the wild-type or the transfectants were obtained by stepwise MTX selection as described (19).

DNA manipulations

Chromosomes in agarose blocks were resolved by trans-alternative field electrophoresis (TAFE, Beckman) as described previously (28). Southern blots, hybridization and washing conditions were done following standard protocols (29). The *ptr1*, *dhfr-ts*, *neo* and *hyg* probes were obtained by the polymerase chain reaction. The R locus probe corresponds to a 2.5 kb *Bgl*II–*Bgl*II fragment located 1 kb downstream of the *L.tarentolae dhfr-ts* gene. Wild-type *L.tarentolae* promastigotes were transfected by electroporation as reported previously (8). Selections were done with 40 μg/ml G418 (Gibco-BRL), and 100 μg/ml hygromycin B.

DNA constructs

The *dhfr-ts* gene of *L.tarentolae* was cloned by screening a cosmid library of partially *Sau*3AI digested total DNA of *L.tarentolae* (21) with a *L.major dhfr-ts* probe. A cosmid called cL-dhfr was isolated and *dhfr-ts* was recovered as part a 6 kb *Sal*I–*Sal*I fragment. Subcloning into the *Leishmania* expression vector pSPY*hyg* (30) produced the plasmid pSPY*hyg* /LT-*dhfr*. Plasmid pSPY*hyg*-α was also used to clone the 2.1 kb *Eco*RI–*Eco*RV *dhfr-ts* fragment of *L.major* and the 6 kb *Sal*I–*Sal*I *dhfr-ts* fragment from the R-circle found in the *L.tarentolae ptr1* null mutant strain selected for MTX resistance.

To integrate the *dhfr-ts* gene into the H locus of *L.tarentolae*, a 2.3 kb *Xho*I–*Xho*I fragment containg *ptr1* was cloned into the vector pGEM3. Digestion with *Apa*I and *Sma*I produced a *ptr1* deletion in which the 7 kb *Hpa*I–*Hin*dIII *dhfr-ts-hyg* expression cassette from plasmid pSPY*hyg* /LT-*dhfr* was integrated as a blunt end fragment. Subsequently a linear 8 kb *Xba*I–*Hin*dIII fragment was isolated and transfected into TarII wild-type cells. Mutants carrying the *dhfr-ts-hyg* cassette within a *ptr1* deletion in the H locus were isolated after selection for hygromycin resistance and named RH (Fig. 2B). To inactivate the remaining *ptr1* allele of the RH strain, we used a 3.3 kb *Xho*I–*Xho*I fragment described previously (27) in which the *ptr1* gene was interrupted by the *neo* gene (Fig. 2C).

To obtain the mutant strain HR with a single *ptr1* copy replacing one *dhfr-ts* allele at the R locus of *L.tarentolae*, the 6 kb *Sal*I–*Sal*I *dhfr-ts* fragment was subcloned into the vector pGEM3. A 1.9 kb *Hpa*I–*Sma*I fragment containing *dhfr-ts* was deleted and replaced by the 3.3 kb *Eco*RV–*Hin*dIII *ptr1-neo* expression cassette from plasmid pSPY*neo/Xho-ptr1* (8). A 7 kb *Pvu*II–*Pvu*II fragment with the *ptr1-neo* cassette replacing *dhfr-ts* was transfected into the *ptr1* null mutant TarII *ptr1Yhyg*.2. The mutant HR (Fig. 2D) was isolated by selection for G418 resistance.

DHFR enzymatic activity

Supernatant from homogenates of *L.tarentolae* promastigotes were prepared and DHFR activity in these extracts was assayed as described previously (31). Dihydrofolate concentration was 300 μM in all assays and in the inhibition assays MTX concentrations were between 0 and 50 nM. NADPH concentration was kept constant at 100 μM.

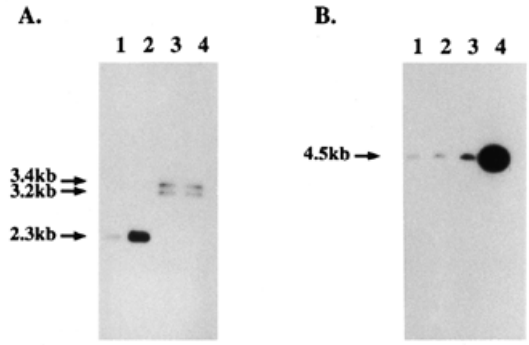

Figure 1. Amplification of *dhfr-ts* in MTX resistant *L.tarentolae*. Equal amounts of genomic DNAs from *L.tarentolae* were digested by *Xho*I and separated on an agarose gel. Southern blots were hybridized with a *ptr1* probe (**A**), and a *dhfr-ts* probe (**B**). The 2.3 kb fragment corresponds to the size of the wild-type *ptr1* allele while the 3.2 and 3.4 kb bands correspond to *ptr1* interrupted by neomycin and hygromycin phosphotransferases in the *ptr1* null mutant. The 4.5 kb fragment corresponds to the *dhfr-ts* gene. Lane 1, *L.tarentolae* wild-type; lane 2, *L.tarentolae* selected for MTX resistance; lane 3, *L.tarentolae ptr1* null mutant; lane 4, *L.tarentolae ptr1* null mutant selected for MTX resistance.

Transport studies

Accumulation of MTX was measured as described (19,21). Tritium labeled MTX (23.6 Ci/mmol) was purchased from Moravek Biochemicals. Transport studies were done using 500 nM of MTX.

RESULTS

Amplification of *dhfr-ts* in *L.tarentolae*

Although along the years, we have selected over 100 *L.tarentolae* mutants for MTX resistance, we have never observed *dhfr-ts* amplification in that species. In several mutants, however, the H locus encoded *ptr1* gene was amplified (19,24–26). We were interested to test how a cell without *ptr1*, which is now hypersensitive to MTX, would respond to MTX selection and in particular whether *dhfr-ts* would be amplified. A *ptr1 L.tarentolae* null mutant already available (27) was therefore selected for MTX resistance in a step by step procedure until it became resistant to 250 μM MTX. The DNAs of wild-type and resistant mutants were isolated and analyzed by Southern blots using *ptr1* and *dhfr-ts* probes. Hybridization with the *ptr1* probe confirmed that we were working with the null mutant as no wild-type *ptr1* allele (a 2.3 kb *Xho*I–*Xho*I restriction fragment) was present and the two novel bands correspond to the integration of the *neo* and *hyg* selectable markers into both *ptr1* alleles (Fig. 1A, lanes 3 and 4). Selection for MTX resistance in wild-type cells led to *ptr1* amplification as extensively observed before, but not to *dhfr-ts* amplification (Fig. 1A and B, lane 2). However, the *dhfr-ts* gene was amplified in a *L.tarentolae ptr1* null mutant selected for MTX resistance (Fig. 1B, lane 4). Amplification of the *dhfr-ts* locus was in fact observed in five out of five independent *ptr1* null mutants selected for MTX resistance (not

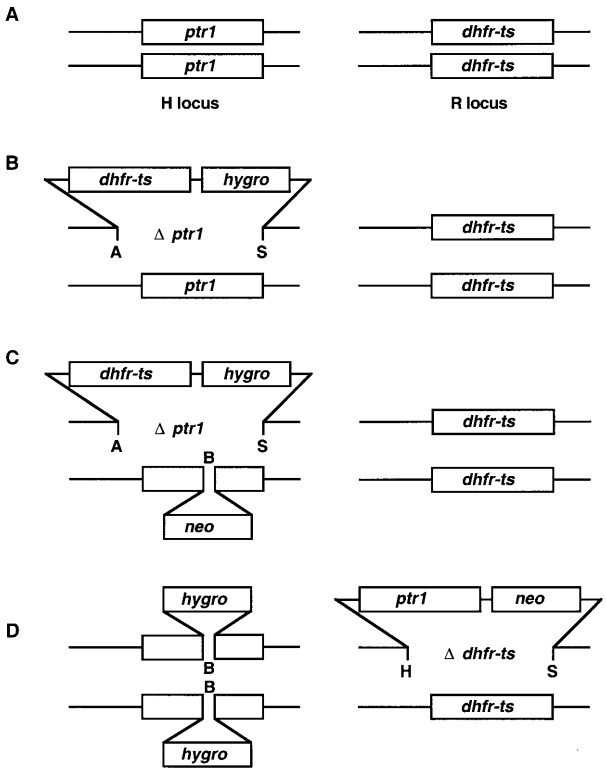

Figure 2. Generation of parasite recombinant strains to test the role of the resistance gene and the locus on gene amplification frequency. Part of the H and R locus of *L.tarentolae* wild-type cells is shown (**A**). The RH (**B**), RH-neo (**C**) and HR (**D**) strains were generated by permuting the *ptr1* and *dhfr-ts* genes at the level of the R and H loci. A, *Apa*I; B, *Bam*HI; H, *Hpa*I; S, *Sma*I.

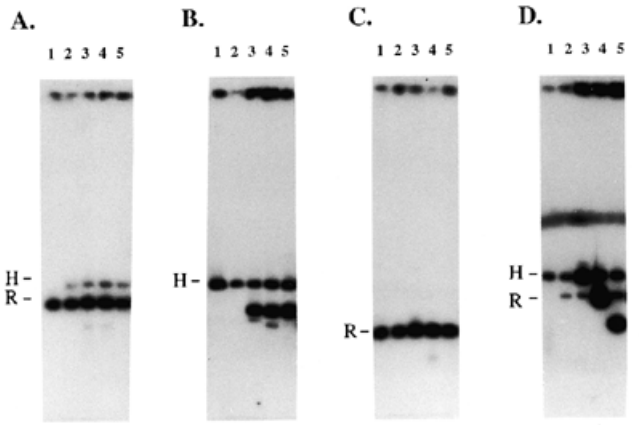

Figure 3. Gene amplification in the strains RH and HR selected for MTX resistance. Chromosomes from the RH strains (**A** and **B**) and HR strain (**C** and **D**) were separated by TAFE and Southern blots were hybridized to a *dhfr-ts* (A and C) or *ptr1* (B and D) probes. H corresponds to the H locus while R corresponds to the R locus. The uniform band above the H locus in (D) is due to background.

shown). The amplicon was circular as it was possible to isolate it by standard plasmid mini-preparation (32). This suggests that once a preferred resistance mechanism is unavailable (e.g. *ptr1* amplification), a secondary one (*dhfr-ts* amplification) will take over.

Role of the resistance gene and the locus on gene amplification frequency

The above results indicated that there are no major obstacles for observing *dhfr-ts* amplification in *L.tarentolae* selected for MTX resistance. To try to explain the lack of *dhfr-ts* amplification in *L.tarentolae* wild-type cells selected for MTX resistance, we tested the role of the locus on gene amplification frequency. The H locus is flanked by several homologous repeated sequences (24) and we have shown that the presence of repeats surrounding a resistance gene will determine the length and type of amplicon formed (25). The high frequency of H locus amplification after MTX selection may therefore be due to the availability of several homologous repeated sequences. The *dhfr-ts* gene present on the R locus in *L.major* is flanked by direct repeats (4) but we cannot exclude that these repeats are absent or mutated in *L.tarentolae* hence explaining the low frequency of *dhfr-ts* amplification in the latter species. We therefore engineered a series of recombinant *Leishmania* cells to test the role of the locus on gene amplification frequency (Fig. 2).

We first attempted to integrate the *dhfr-ts* gene into the H locus by replacing a *ptr1* allele. Transfection of the appropriate construct and selection with hygromycin B gave rise to the RH strain (Fig. 2B). Hybridization to a chromosome sized blot confirmed that the *dhfr-ts* probe now recognizes the 500 kb chromosome (R locus) as well as the 800 kb chromosome (site of the H locus) (Fig. 3A, lane 2). The proper integration of the *dhfr-ts* gene into the H locus was also confirmed by Southern blot analysis of DNA digested with *Hin*dIII and *Xba*I (not shown). Ten independent cultures of an RH clone were selected for resistance to 50 μM MTX, a relatively low concentration of MTX consisting into a concentration 2-fold higher than the EC50 of the cell. The resistant clones were tested for gene amplification by hybridization studies using *dhfr-ts* and *ptr1* probes. Ten out of the 10 mutants showed *ptr1* amplification (Fig. 3B lanes 3–5 and not shown) but none had *dhfr-ts* gene amplification (Fig. 3A lanes 3–5) despite that there were three copies of *dhfr-ts* for one copy of *ptr1* and that one copy of *dhfr-ts* was flanked by the H locus repeated sequences required to be amplified efficiently.

To test further the role of the resistance gene, we compared the frequency of *dhfr-ts* and *ptr1* amplification when both genes were present on the 500 kb chromosome R locus. A construct targeting the *ptr1* gene to the R locus was electroporated into the *L.tarentolae ptr1* null mutant and selected for G418 resistance to generate the HR strain (Fig. 2D). Hybridization to a chromosome-sized blot confirmed the localization of *ptr1* at the 500 kb R locus chromosome (Fig. 3D, lane 2). Ten independent cultures of a clone with the proper integration were selected for resistance at 50 μM, but none had *dhfr-ts* amplification (Fig. 3C, lanes 3–5) while all of them had *ptr1* amplification (Fig. 3D, lanes 3–5). The *ptr1* linear amplicons derived from the R locus differed in size. Since the H locus *ptr1* alleles were inactivated, the amplified *ptr1* gene in the HR mutants was inevitably coming from the R locus. This was indeed confirmed by hybridization with an R

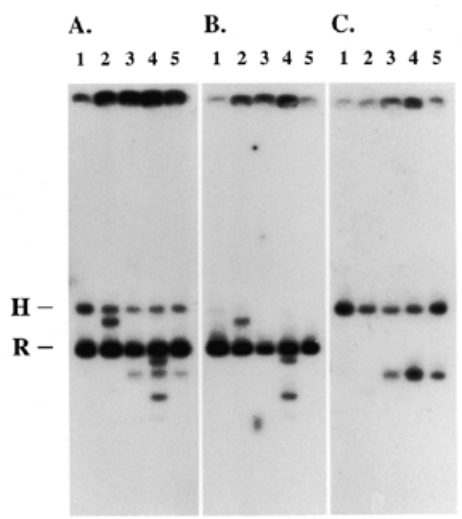

Figure 4. Amplification of *dhfr-ts* in the RH-neo strain. The RH-neo strain (see Fig. 2C) with no functional *ptr1* was selected for MTX resistance and chromosomes from a selection of the mutants were separated by TAFE and hybridized with a *dhfr-ts* probe (**A**), an R locus probe (see Materials and Methods) (**B**) and an H locus probe (the *hyg* gene, see Fig. 2C) (**C**). Lane 1, unselected RH-neo; lanes 2–5, independent RH-neo strains selected for MTX resistance.

locus probe (not shown). The analysis of these mutants further confirmed that the R region can be amplified in *L.tarentolae* MTX resistant mutants.

In order to look more critically at the role of the locus we have inactivated the remaining intact *ptr1* allele in the RH strain by introducing a *ptr1-neo* cassette (27). This led to the strain RH-neo (Fig. 2C). The integration of the *neo* and *hyg* markers at the H locus was confirmed by hybridization of Southern blots (not shown). This strain became hypersensitive to MTX as no functional *ptr1* was available. RH-neo was selected for resistance to 50 μM MTX and the analysis of 10 independent mutants indicated that the *dhfr-ts* gene was amplified in nine of these mutants (Fig. 4 and not shown). The *dhfr-ts* gene as part of the R locus was amplified in six mutants (Fig. 4B, and not shown) while the same gene as part of the H locus was amplified in nine mutants (Fig. 4C, and not shown) with several mutants having the *dhfr-ts* gene amplified from both loci at the same time (e.g. Fig. 4, lane 4). As there is only one H locus *dhfr-ts* allele compared to the two R locus *dhfr-ts* alleles it is tempting to speculate that the locus itself is also important for gene amplification frequency, the same gene being three times more susceptible to amplification from the H locus than the R locus. Consistent with what we have observed previously (26), the first event of gene amplification is the formation of linear amplicons (Figs 3 and 4) and remarkably the H locus derived linear amplicons were very homogeneous in size while the R locus derived amplicons varied in size (Figs 3 and 4).

Transfection studies of the *dhfr-ts* gene

If both the *ptr1* and *dhfr-ts* genes are present, we will only observe *ptr1* gene amplification upon MTX selection in *L.tarentolae*. To test whether *ptr1* is a better drug resistance gene, we cloned the *L.tarentolae dhfr-ts* and *ptr1* genes by screening a genomic bank cloned in the vector cLHyg (33). As reported, transfection of the *ptr1* gene was conferring a high level of resistance to MTX (8,9) while, surprisingly, the *L.tarentolae dhfr-ts* gene was conferring only low level MTX resistance in SDM-79 medium (Fig. 5A). Although low, the resistance provided by *dhfr-ts* is sufficient to have expected amplification of the gene during the first step of MTX selection. Point mutations in DHFR is a common resistance mechanism to antifolates and this has been described at least once in *L.major* DHFR (22). We therefore cloned the *dhfr-ts* gene present on the amplified circle of *L.tarentolae ptr1* null mutant selected for MTX resistance. Transfection of this gene, however, failed to show more resistance than cells transfected with the wild-type allele (Fig. 5A). It is possible that the *L.tarentolae* DHFR-TS gives lower resistance to MTX than the *L.major* gene. The *L.major dhfr-ts* was isolated, cloned into a *Leishmania* expression vector and transfected into *L.tarentolae*. The *L.major dhfr-ts* gene confers the same resistance level as the *L.tarentolae* gene (Fig. 5A). The above results were obtained in SDM-79, a medium rich in folates. As folate concentration modulates resistance to MTX (2,4,34), we tested the DHFR mediated resistance levels of *L.tarentolae* transfectants in M199 medium, a medium containing less folates. As expected, wild-type cells were more susceptible to MTX in M-199 medium (Fig. 5A and C) and the increase in resistance mediated by *dhfr-ts* transfection was considerably higher in the latter medium although still not to the levels (between 35 and >100-fold) reported in the literature (9,22) (Fig. 5C). As *L.major* mutants exhibiting *dhfr-ts* amplification were selected in M-199 medium, we selected *L.tarentolae* MTX resistant mutants in M-199 medium. Analysis of five independent mutants indicated however that *ptr1*, but not *dhfr-ts*, was amplified in all the mutants (not shown). Nevertheless, by simply changing the growth medium, the resistance mediated by DHFR-TS is much more pronounced (Fig. 5A and C). It is also possible that species specific factors could account for the difference in resistance levels provided by *dhfr-ts*- transfection in *L.tarentolae* and *L.major*. The *L.tarentolae* and *L.major dhfr-ts* gene were therefore transfected into *L.major* and those transfectants were then challenged with MTX. We observed, however, the same low level resistance in *L.major* when measured in SDM-79 medium (Fig. 5B) while resistance observed was sensibly higher when tested in the M-199 medium (Fig. 5D).

The transfection of the *dhfr-ts* gene in *L.tarentolae* can confer up to 10-fold increase in resistance to MTX in M-199 medium which is much higher than in SDM-79 but still less than the value described in the literature (9,22). To exclude that the low DHFR-TS activity measured in our cells was due to non-optimal expression in our vector, we measured DHFR activity in the three *dhfr-ts L.tarentolae* lines transfected with different *dhfr-ts* (originating from *L.tarentolae*, *L.major* and from an amplicon of a resistant mutant) constructs. The DHFR activity was measured from the crude extracts of wild-type cells and determined to be 2.1 nmol/min/mg which is similar to other DHFR activities determined from crude extracts of other species of *Leishmania* (12,22,31). The DHFR activity of the crude extracts of the three transfected lines was 15-fold higher than wild-type cells (Fig. 6) with specific activities around 30 nmol/min/mg. These values are similar to those reported from

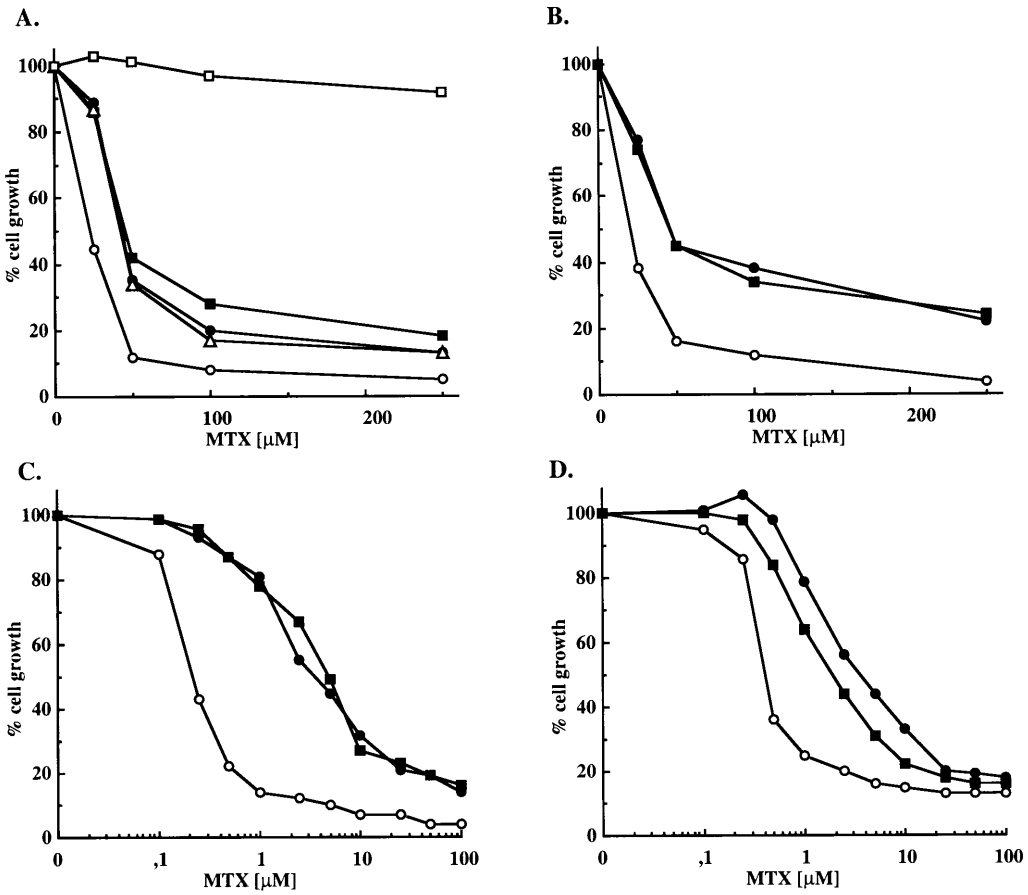

Figure 5. Resistance phenotype of *dhfr-ts* transfectants. The growth of *L.tarentolae dhfr-ts* transfectants (**A** and **C**) and of *L.major dhfr-ts* transfectants (**B** and **D**) was evaluated in SDM-79 medium (A and B) or M199 medium (C and D) with increasing concentrations of MTX. Open circle, *L.tarentolae* transfected with a control *hyg* plasmid; closed circle, cells transfected with the *L.tarentolae dhfr-ts* gene; closed square, cells transfected with the *L.major dhfr-ts* gene; triangle, cells transfected with the *dhfr-ts* gene isolated from a circular amplicon present in the *L.tarentolae* MTX resistant *ptr1* null mutant, (Fig. 1, lane 4); open square, *L.tarentolae* transfected with *ptr1*.

L.major cells in which *dhfr-ts* was amplified (22). The DHFR activity of the extracts was inhibited in a similar way by MTX (Fig. 6).

Other mechanism of MTX resistance

Since in our hands *dhfr-ts* confers relatively low level resistance to MTX, we hypothesized that other mutations, in addition to *dhfr-ts* amplification (Fig. 1), were likely to be present in the *ptr1* null mutant selected for MTX resistance. As most *Leishmania* cells resistant to MTX exhibited a mutant transport phenotype, we looked at the transport of MTX in the *ptr1* null mutants selected for MTX resistance. All the mutants tested showed no measurable accumulation of radioactive MTX (Fig. 7, and not shown) indicating that in addition to *dhfr-ts* amplification, the mutants require a reduction in the uptake of the drug in order to resist MTX.

DISCUSSION

Resistance to the model antifolate drug MTX has been extensively studied in the parasite *Leishmania*. Amplification of the target gene *dhfr-ts* occurs in MTX resistant *Leishmania* although this has only been seen in *L.major* (6,17,22) and not in other *Leishmania* species (3). Amplification of the short chain dehydrogenase *ptr1* is more frequently observed following MTX selection. The main role of PTR1 is to reduce pterins but when overexpressed it can reduce sufficient dihydrofolates into tetrahydrofolates so that when *dhfr-ts* is inhibited by MTX, cells will nevertheless continue to grow (2,10,12,35). All *Leishmania* species also resist MTX by reducing its uptake through mutations in its common folate/MTX transporter (17–19).

Amplification of *dhfr-ts* is a common mechanism of MTX resistance in several type of cells (3), and we found intriguing the lack of *dhfr-ts* amplification in *L.tarentolae* MTX resistant mutants. This was therefore investigated further. Upon MTX selection, *L.tarentolae* almost invariably will amplify its *ptr1* gene (19,25,26) and transport mutations are also frequently encountered (19,21). We investigated the outcome of the selection of a *ptr1-/ptr1-* null mutant for MTX resistance. In five independent *ptr1-/ptr1-* strains selected for MTX resistance we observed *dhfr-ts* amplification (Fig. 1 and not shown). This indicates that once a preferred resistance mechanism is

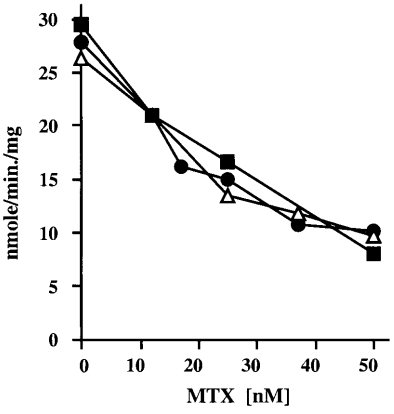

Figure 6. DHFR-TS enzymatic activity and inhibition by MTX. The DHFR enzymatic activity of crude extracts of *Leishmania* cells was measured using 300 μM of dihydrofolate and 100 μM NADPH. Open circle, *L.tarentolae* transfected with a control *neo* plasmid; closed circle, cells transfected with the *L.tarentolae dhfr-ts* gene; square, cells transfected with the *L.major dhfr-ts* gene; triangle, cells transfected with the *dhfr-ts* gene isolated from a circular amplicon present in the mutant shown in Figure 1, lane 4.

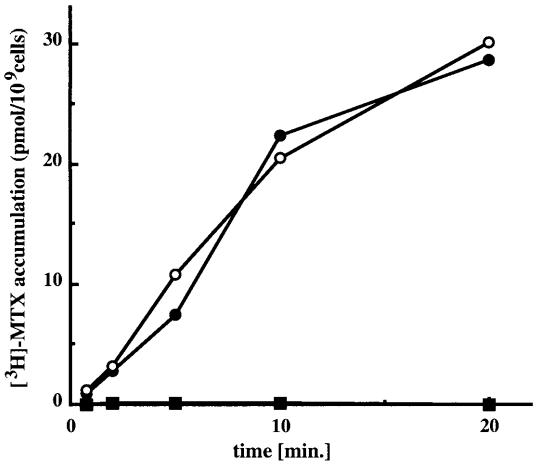

Figure 7. Transport studies of ^{3}[H]-MTX in *Leishmania* cells. Transport studies were done as described in Materials and Methods. Open circle, *L.tarentolae* wild-type cells; closed circle, *L.tarentolae ptr1*null mutant; square, *L.tarentolae ptr1*null mutant resistant to MTX.

unavailable to the cell a second one will arise. To be successful in *Leishmania* antifolate chemotherapy it would therefore be necessary to target both PTR1 and DHFR-TS and lead compounds against these two targets are now available (36).

The results presented in this study show that there is no major obstacle for *dhfr-ts* amplification in *L.tarentolae*. We have studied extensively the mechanisms of gene amplification in *Leishmania* and showed that amplicons are usually formed at the level of homologous repeated sequences (24,25). We know that the *L.tarentolae* H locus is flanked by several repeated sequences but the *L.tarentolae* R locus has not been characterized. The *L.major* R locus is flanked by repeated sequences (4). To test the effect of the locus, we introduced a *dhfr-ts* allele in the H locus replacing one copy of *ptr1*. After selection with MTX, we could only detect *ptr1* amplification (Fig. 3B, lanes 3–5). Similarly, when the *ptr1* gene was absent from its endogenous locus but present at the R locus, we observed, after MTX selection, *ptr1*-R locus-derived gene amplification (Fig. 3). As we found out that *ptr1* is a better MTX resistance gene than *dhfr-ts* (Fig. 5) the latter results are not overwhelmingly surprising and indicate that the resistance gene is crucial for selecting for gene amplification. In order to test the effect of the locus in more detail, we have inactivated the remaining *ptr1* allele in the RH transfectant (Fig. 2C). Selection for MTX resistance revealed that the locus is also important in determining the frequency of gene amplification. Indeed, when present on the H locus, the *dhfr-ts* gene was amplified three times more (considering that there is one copy at the H locus and two copies at the R locus) than when present on the R locus (Fig. 4). The first step in gene amplification after MTX selection is the formation of linear amplicons (Figs 3 and 4) (26,37). As noted previously (26), the H locus derived amplicons are very homogenous in size while the R locus amplicons differ greatly in size (Figs 3 and 4). It is possible that specialized sequences are implicated in the formation of H locus amplicons and that the presence of these sequences may increase the likelihood of a locus to be amplified hence explaining the higher rate of *dhfr-ts* amplification when part of the H locus. In yeast, the chromosomal position of the *dhfr* gene was also shown to exhibit considerable effect on the rate of *dhfr* amplification upon MTX selection (38).

The resistance gene itself is obviously important for gene amplification. Upon selection for MTX resistance of a *ptr1* null mutant in which a novel *ptr1* copy is integrated at the R locus, we exclusively observed *ptr1* amplicons derived from the R locus but no R locus amplification containing *dhfr-ts*. Under our experimental conditions, DHFR-TS confers much lower resistance than *ptr1* and we believe this is a sufficient explanation for not observing its amplification. The DHFR enzymatic activity determined from crude extracts seems however to have comparable activities to other characterized DHFR-TS (12,22,31) and the inhibition profile with MTX is similar (Fig. 6). The reason why *dhfr-ts* confers low level resistance is unknown but does not seem to be due to species specific factors (Fig. 5). We cannot exclude, however, that by prolonged culture in SDM-79 we have changed some aspects of folate metabolism that may influence DHFR-TS induced resistance levels. In conclusion, this paper shows that both the resistance gene itself and the locus are important for selection of gene amplification events. Finally, amplification of *dhfr-ts* is possible in *L.tarentolae* and indicates that once a preferred resistance mechanism is unavailable, a second one will take over.

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